EXHIBIT A

DECLARATION OF YI LI

I, YI LI, HEREBY DECLARE AS FOLLOWS:

1. I am a co-inventor of the subject matter disclosed and claimed in the above-referenced patent application.

2. I understand that the United States Patent and Trademark Office (PTO) Examiner in charge of examining my application for patent has taken the position that my patent application discloses only prophetic examples that, while fully described, have not been shown to be executed with confirmatory data.

3. I have demonstrated in several working examples that gene cassettes incorporated into plants can be 100% excised from the transgenic plant by employing the methods disclosed in the referenced patent application.

4. Briefly, I have combined LoxP (34bp) and/or FRT (48bp) recognition sequences in direct orientation as flanking sites for a FLP and/or Cre recombinase gene that is engineered to express in pollinating plants when an operatively linked promoter is activated. The gene cassette, including a GUS reporter gene, was constructed using the standard techniques detailed in the paper provided in Attachment A. Transformed tobacco plants were cultivated and analyzed before and after pollination (immature and mature plants). PCR and other analyses showed that the reporter gene and the sequences between the flanking recombinase excision sites were 100% deleted, leaving only a short nonfunctional DNA fragment (could be as short as 86 bp) that was not excised.

5. Exhibit B is a simplified cartoon and description of results using the engineered cassette. Figure 3 in Exhibit B provides molecular evidence for deletion of all functional gene cassettes from pollen grains.

6. All statements made in this Declaration of my own knowledge are true and all statements made in this Declaration on information and belief are believed to be true, and these statements are made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both under U.S.C.§1001 and may jeopardize the validity of this application or any patent issuing thereon.

12/24/03
Date

Yi Li
Yi Li

ATTACHMENT A

Exhibit B

Constructs

A single excision system, either LoxP-Cre or FRT/FLP system is used to delete transgenes from target organs or plants

LoxP ---- Trait Gene ----Marker Genes ---- CRE (recombinase) Gene ---- LoxP

FRT ---- Trait Genes ---- Marker Genes ---- FLP (recombinase) Gene ---- FRT

Both LoxP and FRT have been combined to increase the excision efficiency. When only FLP is used, the excision efficiency was near 100%.

Lox-FRTd ---- Trait Genes ---- Marker Genes ---- FLP (recombinase) Gene ---- LoxP-FRT

Constructs in Working Examples

35SGN: -- (T-DNA LB) -- (35S-GUS-NOS) --- (35S-NPTII-NOS) --- (T-DNA-RB)---

LFFGN: -- (T-DNA LB) -- (Lox-FRT) --- (35S-GUS-NPTII) --- (BGP-FLP) --- (LoxP-FRT) -- (T-DNA-RB)--

--35S Promoter: 35 S CaMV gene promoters, constitutively and globally active

-- GUS: A reporter gene whose gene product can be conveniently assayed. Serving two functions: a) being used as an ideal tool to monitor the excision of transgenes in pollen and other organs, and b) representing a trait gene.

-- NPTII: Kanamycin resistance gene for selection of transgenic plants

-- BGP: A pollen specific gene promoter

-- FLP: A recombinase gene whose protein product can recognizes FRT sequences and delete the DNA sequence in between.

-- T-DNA-LB: Transfer DNA left border

-- T-DNA-LB: Transfer DNA right border

Results

Results are tablulated in Table 1 showing that there was no recombination resulting in the presence of the marker gene.

Table 1. Efficiency of Transgene Deletion in Pollen with "Naturalizer" Technology

Transgenic line	GUS (+)	GUS(-)	Deletion Efficiency
35-GUS without "naturalizer" gene cassette*:			
1 male, 1 copy GUS) x wildtype (female)	3,054	3,301**	0%
35-GUS with "naturalizer" gene cassette:			
1 (male, 1 copy GUS) x wildtype (female)	0	11,083	100%
2 (male, 1 copy GUS) x wildtype (female)	0	16,368	100%
3 (male, 1 copy GUS) x wildtype (female)	0	25,989	100%
4 (male, 2 or more copies GUS) x wildtype (female)	0	15,876	100%
5 (male, 2 or more copies GUS) x wildtype (female)	0	13,045	100%

* "Naturalizer" gene cassette: Lox-FRT ---- Trait Genes ---- Marker Genes ---- FLP (recombinase) Gene ---- LoxP-FRT
**GUS (-) seeds in the 35S GUS line without the "naturalizer" gene cassette are due to genetic segregation of the GUS gene because the transgenic plant is heterozygous and contains a single copy of the GUS gene.

These results are further confirmed in Figs 1A,B, and C and in Fig. 2.

Fig. 1. Deletion of the GUS gene (indicated by blue color stain) in specifically in pollen of transgenic plants

A, B, C and D: The GUS gene was present an expressed in every tissue and organs: leaf (**A**), stem (**B**), root (**C**) and flower (**D**) of the LFFGN transgenic plants. **E and F**: The GUS gene was present and expressed in young pollen grains (**E**) and mature pollen grains (**F**) of a 35SGN transgenic plant. **G and H:** The GUS gene was present and expressed in young pollen grains but deleted from mature pollen grains of a LFFGN plant.

Fig. 2. Deletion of the GUS gene (indicated by blue color stain) in specifically in pollen of transgenic plants

A: 75% seeds from a self-pollinated 35SGN control plant exhibited contain an express the GUS gene (blue color). **B:** 50% seeds from a self-pollinated LFFGN plaint contain and express the GUS gene because 50% of LFFGN ovules contain the GUS gene although 100% LFFGN pollen grains have no functional transgenes. **C:** 50% hybrid seeds from a cross of 35SGN plant X wildtype plant contain and express the GUS gene (blue color). **B:** No hybrid seed from a cross of LFFGN plant X wildtype plant contains the GUS gene because LFFGN pollen grains are 100% free of functional transgenes. Consistently, the seedlings derived from seeds in Fig. C show blue color **(E)** while seedlings derived from seeds of Fig. D contain no GUS gene **(F)**.

Deletion of All Functional Transgenes

Molecular Evidence of Deletion of All Functional Transgenes from Pollen Grains is shown in Fig. 3.

MM: Molecular size marker
Lane 1: No 7.3 kb band (the transgenes or foreign DNA sequence) was observed in the wildtype plant.
Lane 2: No. 0.2 kb band (the remaining foreign DNA sequence after excision) was observed in the wildtype plant.
Lane 3: A 7.3 Kb band (the transgenes or foreign DNA sequence) was observed in Transgenic Line 1.
Lane 4: A 0.2 kb band (the remaining foreign DNA sequence after excision) was observed in Transgenic Line 2.
Lane 5: A 7.3 kb band (the transgenes or foreign DNA sequence) was observed in Transgenic Line 2.
Lane 6: A 0.2 kb band (the remaining foreign DNA sequence after excision) was observed in progeny of Transgenic
Line 2.
Lane 7: A 7.3 kb band (the transgenes or foreign DNA sequence) was observed in Transgenic Line 3.
Lane 8: A 0.2 kb band (the remaining foreign DNA sequence after excision) was observed in progeny of Transgenic
Line 3.
Lane 9: A 7.3 kb band (the transgenes or foreign DNA sequence) was observed in Transgenic Line 4.
Lane 10: A 0.2 kb band (the remaining foreign DNA sequence after excision) was observed in progeny of Transgenic
Line 4.
Lane 11: A 7.3 kb band (the transgenes or foreign DNA sequence) was observed in Transgenic Line 5.
Lane 12: A 0.2 kb band (the remaining foreign DNA sequence after excision) was observed in progeny of Transgenic
Line 5.

Fig.1.

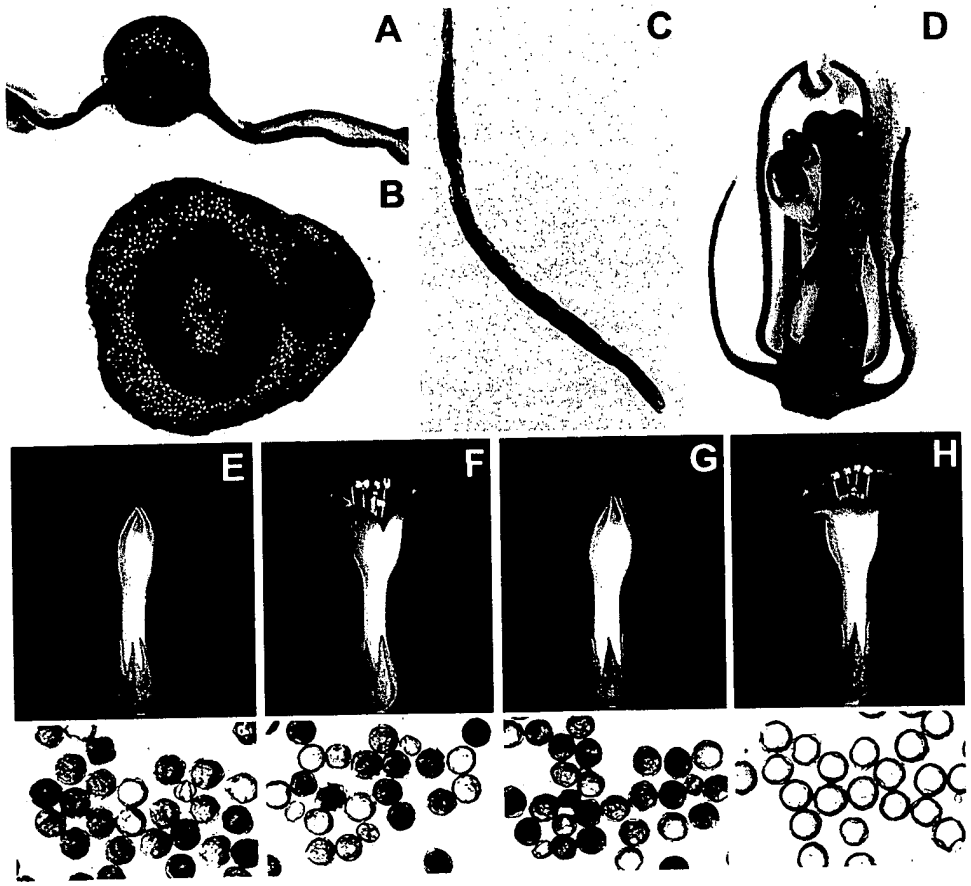

Fig.2.

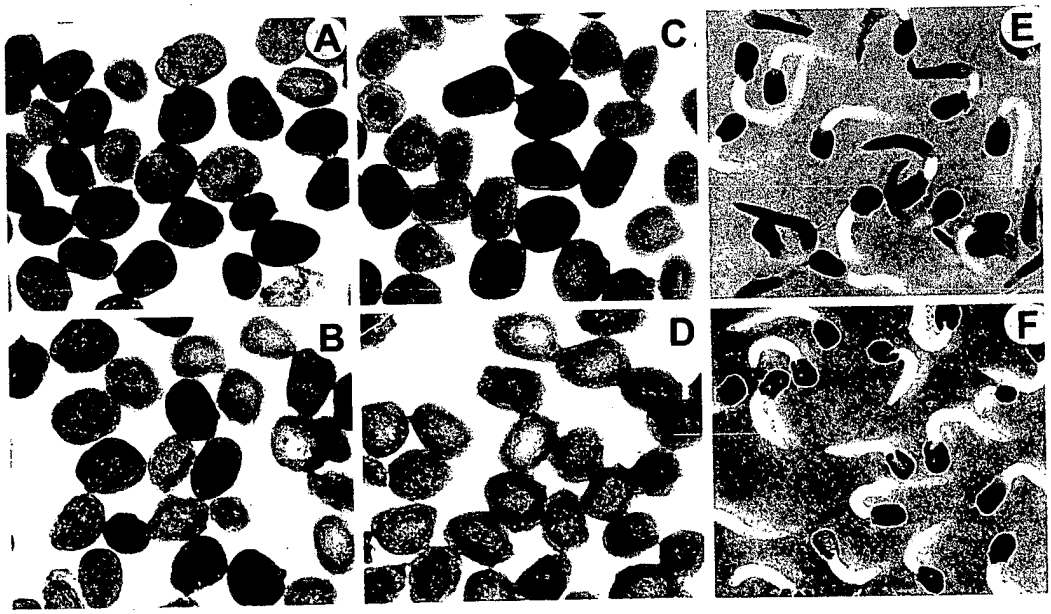

Fig.3.

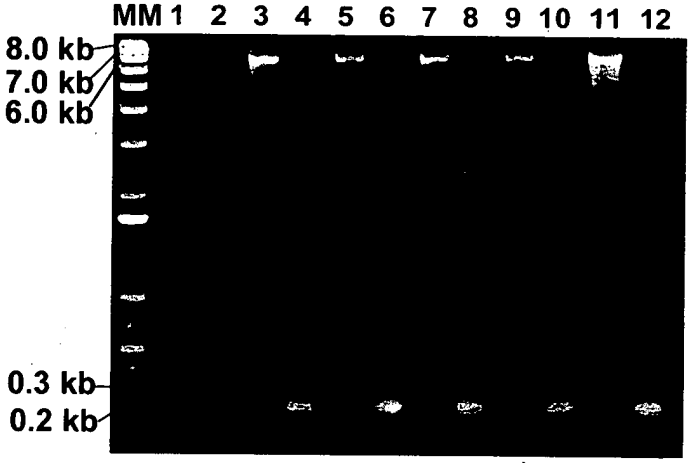

Fig. 1

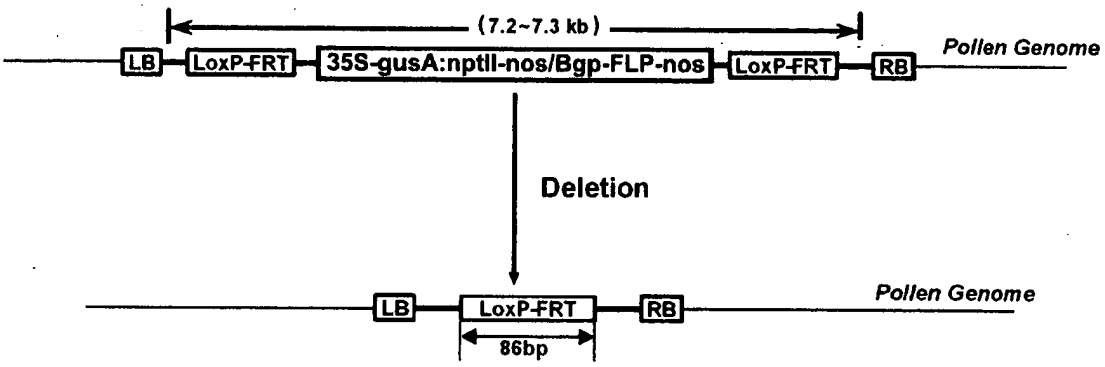
(7.2~7.3 kb)
LB
LoxP-FRT
35S-gusA:nptII-nos/Bgp-FLP-nos
LoxP-FRT
RB
Pollen Genome
Deletion
LB
LoxP-FRT
RB
Pollen Genome
86bp

Fig. 2


MM
F2
F4
F6
F9
F11
WT
14.0 kb
2.2 kb

ATTACHMENT B

Manuscript submitted:

A highly efficient system to remove foreign genes from the pollen genome of transgenic plants

Keming Luo[1,2,3], Hui Duan[1,3], Degang Zhao[1,3], Yanhong Wu[1], Xiangning Jiang[1], Yan Pei[2], Aigong He[1], Richard McAvoy[1] & Yi Li[1*]

Abstract:

Although the potentially negative environmental impact of GM pollen has been of concern, none of the existing technologies to address this problem is broadly applicable for field crops. Here we report a novel "gene-deletor" system based on a combined use of FLP/*FRT* and Cre/*loxP* recombination systems. With pollen specific expression of the FLP or Cre recombinase gene, the "gene-deletor" system we have developed is exceptionally efficient in deleting all functional transgenes from the pollen genome of transgenic tobacco plants. This highly efficient "gene-deletor" system may provide a tool to address the environmental impact GM pollen and also with minor modifications the technology might be useful to produce non-transgenic seeds or fruits from transgenic crops or to protect proprietary transgenic plant technologies. Furthermore, the "gene-deletor" technology might be extended to animal systems for deletion of transgenes at desirable times and for improvement of the efficiency of gene knockouts.

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[1]Department of Plant Science, University of Connecticut, Storrs, CT 06269, USA.
[2]Biotechnology Center, Southwest Agricultural University, Chongqing, P. R. China.
[3]These authors contributed equally to this work and are considered first authors.
KL and XJ are visiting scientists from Southwest Agriculture University, Chongqing, and Beijing Forestry University, Beijing, P. R. China, respectively.

*Correspondence author (yi.li@uconn.edu).

Plants produce large amounts of pollen that can disperse over long distances via insects and wind. Gene transfer through pollination routinely occurs within the major world

crops, such as wheat, rice, maize, soybean, barley, and cotton, and also between these crops and their wild relatives[1]. To date, more than 52 million hectares of transgenic crops have been grown worldwide. In the case of canola, one of the first GM crops, Rieger et al. [2] have demonstrated that cross-pollination between commercial canola fields can occur over considerable distances. In addition, pollen of some GM crops may cause adverse effects on non-target organisms[3]. The claims that Bt corn pollen killed larval monarch butterfly[4] and that transgenic maize genes had introgressed to wildtype maize in remote areas of Mexico[5] have raised storms of controversy about GM plants[3, 6]. In recent years, several technologies have been developed to address the pollen-mediated "transgene pollution" problem but none has proved broadly applicable in field conditions[7]. Inserting transgenes into the chloroplast genome appears to be most promising but pollen of some angiosperm and gymnosperm species such as alfalfa, tobacco, pea, rice and conifer contains chloroplasts and transmits chloroplast genes in these species[8]. More recently, it has been shown that DNA is transferred from the chloroplast and integrated into the nucleus at a frequency of one in approximately 16,000 tobacco pollen grains[30]. Furthermore, because of technical difficulties, chloroplast transformation has been achieved only in a very small number of species so far[9].

Site-specific recombination systems such as Cre/*loxP* of phage P1, R/*RS* of *Zygosaccharomyces rouxii* and FLP/*FRT* of *Saccharmyces cerevisiae*, function through interactions of a recombinase with its specific recognition sites[10]. Depending on the orientation of the recombinase recognition sites, intramolecular (excision) or intermolecular (integration) recombination can occur. Intramolecular recombination between directly oriented sites results in excision of the intervening DNA between the two sites and leaves one site behind. Although strategies for removing marker genes (e.g., antibiotic resistance genes) or spacer sequences from the host plant genome using site-specific recombinases have been developed[11,12,13], the possibility of creating non-transgenic organs or plants from transgenic plants has not been addressed[14]. Keenan and Stemmer[14] have recently proposed to use a site-specific recombinase to remove transgenes from specific tissues in crop plants to address safety concerns regarding GM plants but the proposed technology has not been demonstrated experimentally. One of major technical challenges is that excision efficiencies of the existing site-specific recombination systems are generally low in higher plants[12, 13]. Low efficiency of transgene deletion can make the proposed technology little useful for large-scale crop production because even if a small fraction of pollen, seeds or fruits remains transgenic, the total number of pollen grains, seeds or fruits that contain transgenes can enormously

large. A chemical inducible excision for a transformation marker gene recently described by Zuo et al.[15] appears to be highly efficient in a laboratory setting, but uniform application and effective penetration of the chemical to target tissues under field conditions can be technically difficult.

To address the pollen-mediated transgene pollution problem of field-grown GM crops, we designed novel gene cassettes using components of both FLP/*FRT* and Cre/*loxP* recombination systems. With a combined use of the *loxP* (34-bp) and *FRT* (48-bp) recognition sequences as the flanking sites for a FLP or Cre recombinase expressed in pollen, we observed 100% efficiency for automatic deletion of all functional foreign genes from the pollen genome specifically. We named this method the "gene-deletor" technology.

Results

Fig. 1 shows the schematic diagram of the "gene-deletor" cassette with the *loxP-FRT* sequences flanking a pollen specific FLP recombinase gene (*bgp*-FLP-*nos*), a *gusA* reporter gene and the *nptII* (kanamycin resistance) gene (35S-*gusA:nptII-nos*), and the steps involved in transgene removal from the pollen genome. Because most of the T-DNA sequences within the two borders of pBIN19 Ti-plasmid[16] are nonessential for *Agrobacterium* infection[17], we first deleted these sequences and then inserted two copies of *loxP-FRT* fusion sequences (86-bp in each) in direct orientation. Subsequently, a set of restriction enzyme sites was introduced between two of the *loxP-FRT* sequences. We then engineered a pollen-specific *bgp* gene promoter[18]-FLP recombinase[19] gene (*bgp*-FLP-*nos,* abbreviated as BF) and a globally active 35S CaMV gene promoter[21]-*gusA* reporter gene and the *nptII*[20] gene (35S-*gusA:nptII-nos,* abbreviated as GN) fusion into the two *loxP-FRT* sites, producing the pBIN19-LFBF-GN cassette. Because the expression of the FLP gene is expected to occur specifically in pollen, all transgenes including the FLP gene itself will be excised from the pollen genome except for an 86–bp non-protein encoding *loxP-FRT* sequence. The excised DNA sequence, a 7.3-kb fragment consisting of one of the two *loxP-FRT* sites, the *bgp*-FLP-*nos* gene and the 35S-*gusA: nptII-nos* gene in the case of the LFBF-GN plants, should be destroyed by non-specific nucleases in the cell. The *gusA* reporter gene can be used as a convenient marker to monitor the presence or absence of the transgenes in the cell.

To examine the excision efficiency of the LFBF-GN system, more than 35 transgenic tobacco plants were produced. No visible changes in morphology or developmental processes were observed in greenhouse-grown LFBF-GN transgenic plants. The copy number of the LFBF-GN inserts in LFBF-GN lines 2, 4, 6, 9 and 11, was determined using Southern blot analysis.

Fig. 2 shows Southern blot hybridization results of the genomic DNA digested with *Hind III*. Based on the restriction enzyme map (see Fig. 1), the *Hind III*-digested genomic DNA of LFBF-GN transgenic plants should produce two differently sized DNA fragments, a 2.2-kb size-fixed fragment (an internal T-DNA fragment that contains a 0.8-kb FLP coding sequence and an 1.4-kb the *nptII-nos* gene) and another size-variable fragment (a flanking genomic sequence that contains a 0.4-kb FLP coding sequence, 3'-*nos*, *loxP-FRT,* RB and a size-variable DNA fragment beyond RB) that can hybridize with the FLP probe if the plant harbors a single copy of LFBF-GN. If a plant harbors two copies of LFBF-GN, the *Hind III* digestion should produce three DNA fragments that can hybridize with the FLP DNA probe. If a plant harbors three copies of LFBF-GN, the *Hind III* digestion should produce four fragments that can hybridize with the FLP probe. As shown in Fig. 2, *Hind III*-digested genomic DNA from the representative transgenic lines all had the 2.2-kb DNA fragment as expected. In transgenic lines 4, 6 and 9, only one additional band was observed in each, suggesting that these three plants harbored a single LFBF-GN insert. Two additional bands were detected in transgenic line 11, suggesting that the plant contained two copies of the LFBF-GN inserts in the genome. In the case of LFBF-GN transgenic line 2, at least 5 bands were detected, indicating multi-copies of the LFBF-GN inserts in the genome. The results from genetic segregation analysis for both GUS activity and kanamycin resistance of the offspring of these transgenic lines also support the conclusions regarding the LFBF-GN copy number derived from the Southern blot hybridization data (data not shown).

Detailed histochemical analysis for GUS activity of T0 plants and T1 progenies of LFBF-GN lines 2, 4, 6, 9 and 11 was preformed. GUS activity was detected in all tissues and organs of the LFBF-GN plants: leaves, stems, roots and flowers (Fig. 3 A-D), consistent with the fact that the 35S CaMV gene promoter is globally and constitutively active in the plant[21]. The expression pattern of the *gusA* gene in LFBF-GN plants demonstrates that the FLP gene under the control of the *bgp* gene promoter is not active in non-pollen organs. To examine the FLP-mediated excision efficiency of functional transgenes in pollen, we conducted histochemical staining of GUS activity in pollen at different stages of flower development. As shown in Fig. 3E and 3F, GUS activity was observed in both immature and mature pollen grains of the 35S CaMV promoter-*gusA* (35S-*gusA*) plants lacking the "gene-deletor" system. For transgenic LFBF-GN lines 4, 6, 9 and 11 which harbored one or two copies of the transgene inserts, GUS activity was detected in immature pollen but disappeared in mature pollen (Fig. 3G and 3H), suggesting that the *gusA* gene had been deleted from the pollen genome at later stages of pollen development.

To confirm the pollen staining results, we conducted a large number of self- and cross-pollinations between the LFBF-GN (pollen parent) lines and wildtype (seed parent) plants. The number of GUS positive progeny from the crosses was used to calculate the excision efficiency in pollen grains. For the 35S-*gusA* transgenic plants (single copy of the *gusA* gene without the "gene-deletor" system), we observed that 25% of the progeny were GUS negative when self-pollinated, a result consistent with the expected 3:1 simple Mendelian ratio. When pollen from the 35S-*gusA* plants was used to cross with wildtype plants, approximately 50% of the progeny were GUS negative. For LFBF-GN lines 4, 6 and 9 that contained one copy of the transgene insert, we observed that 50% of the progeny were GUS positive when self-pollinated (Table 1). If the pollen grains of the LFBF-GN plants were used to cross with wildtype plants, the progeny were 100% GUS negative. In the case of LFBF-GN lines 4 and 9, more than 20,000 hybrid progeny produced from LFBF-GN (pollen parent) x wildtype (seed parent) crosses were examined but not a single one was GUS positive (Table 1). To determine whether the combined use of the *loxP* and *FRT* sequences also enhanced efficiencies of the Cre recombinase-mediated excision, we replaced the pollen specific *bgp*-FLP gene (abbreviated as BF) in the LFBF-GN construct with a pollen specific *lat52*[23] gene promoter-*Cre* recombinase gene (abbreviated as LC) to produce the LFLC-GN construct. The *lat52* gene promoter cloned from tomato plants is active in pollen specifically[23]. Similar to LFBF-GN plants, two representative LFLC-GN transgenic lines (5 and 8) examined were also 100% efficient in removing transgenes in the pollen genome (Table 1). Furthermore, as expected, LFLC-GN transgenic plants were morphologically normal and transgene excision did not occur in non-pollen organs (date not shown).

At the DNA level, polymerase chain reaction (PCR) and Southern blot hybridization techniques were used to confirm the complete deletion of functional transgenes from pollen of the LFBF-GN transgenic plants. With the genomic DNA isolated from immature pollen of stage 7 flowers[22] of LFBF-GN plants as template DNA and a pair of short oligos annealing to the outside of the two *loxP-FRT* sites but within the two T-DNA border sequences as primers, we obtained a 7.3-kb DNA fragment (Fig. 4A), consistent with the size of the LFBF-GN gene cassette inserted into the host genome. Under the same experimental conditions, if the genomic DNA isolated from the progeny of LFBF-GN lines 4, 6, 9 and 11 (pollen parent) x wildtype (seed parent) crosses was used as template DNA, we observed a 0.2-kb DNA fragment amplified but the 7.3-kb DNA fragment disappeared (Fig. 4A). The 0.2-kb fragment presumably consisted of one copy of the *loxP-FRT* recognition sequence plus T-DNA sequences remained within the two T-DNA borders. This 0.2-kb signal was not evident either

in the LFBF-GN plants before excision or in wildtype plants. However, presumably due to the presence of multiple copies of the LFBF-GN inserts in the genome of LFBF-GN line 2 plants, an incomplete deletion of transgenes occurred, both 7.3-kb and 0.2-kb fragments amplified, when the genomic DNA of LFBF-GN line 2 x wildtype plant was used as template. In the case of LFLC-GN transgenic lines 5 and 8 analyzed, a 7.2-kb DNA fragment, consistent with the size of the LFLC-GN gene cassette, was amplified with PCR using the genomic DNA isolated from the immature pollen as template (Fig. 4A). The 0.2-kb DNA fragment was also the only product when the genomic DNA from the progenies of the LFLC-GN lines 5 and 8 (pollen parent) x wildtype (seed parent) crosses was used as template DNA (Fig. 4A). The identities of the PCR-amplified 7.3-, 7.2- and 0.2-kb DNA fragments from the LFBF-GN, LFLC-GN, LFBF-GN x wildtype, or LFLC-GN x wildtype crosses were confirmed with DNA sequencing analysis. The complete disappearance of the 7.3- or 7.2-kb DNA fragment and the appearance of the 0.2-kb post-excision signal in the progeny of the LFBF-GN x wildtype crosses or LFLC-GN x wildtype crosses further demonstrated that all functional transgenes were deleted and the *loxP-FRT* hybrid system enhanced the FLP or Cre-mediated excision of DNA sequences flanked by two *loxP-FRT* sequences.

Using genomic DNA isolated from individual hybrid plants and population of progeny seedlings derived from the crosses of LFBF-GN lines 4, 6, 9, and 11 (pollen parent) x wildtype (seed parent) plants, we preformed Southern blot analysis to confirm that FLP and other functional transgenes were completely deleted from pollen of these transgenic plants. Fig. 4B shows strong hybridization signals detected in T0 LFBF-GN lines 4 and 6 but no signals detected in hybrid seedlings produced from the crosses of LFBF-GN lines 4, 6, 9 and 11 x wildtype plants when a FLP fragment was used as probe. No hybridization signals were observed when the *gusA-nptII* DNA fragment was used as probe (data not shown). These results provide additional evidence to support that functional transgenes have been completely deleted from the pollen genome of LFBF-GN 4, 6, 9 and 11 plants. Regarding the precision of deletion of transgenes from the host genome, the DNA sequencing analysis revealed that the two 0.2-kb post-excision signals cloned from the LFBF-GN x WT or LFLC-GN x WT hybrid seedlings consisted one copy of the *loxP-FRT* sequence, and the deletion junctions for both FLP- and Cre-recombinases lied within two *loxP-FRT* direct repeats (Fig. 4C). To determine the relationship between expression levels of the FLP gene and the excision efficiency, RT-PCR technique was used to estimate the transcript levels of the FLP gene in pollen grains of LFBF-GN lines 2, 4, 6, 9 and 11. Because little GUS activity was detected in mature pollen from stage 11 and 12 flowers of LFBF-GN plants, onset of expression of the FLP gene likely occurs between stages 9 to 10 of flower development. With mRNA from pollen of stage 10

flowers as template, RT-PCR analysis showed that the FLP transcript levels were high in LFBF-GN lines 4, 6, 9 and 11 but relatively low in line 2 (Fig. 5). Thus, the incomplete excision of the transgenes in pollen of LFBF-GN line 2 could be due to a low level of the *FLP* gene expression. On the other hand, it is also possible that the inefficient excision of the transgenes from the pollen of LFBF-GN line 2 is because of the existence of multi-copies of transgenes in the genome.

Discussion

As presented here, with the combined use of the *loxP* and *FRT* sequences as flanking sites for either FLP or Cre recombinase, we have developed an exceptionally efficient "gene-deletor" technology to remove all functional transgenes from pollen. The pollen-mediated transgene pollution is a serious concern of the scientific community and the public. Developing a technology to produce transgene-free organs such as transgene-free pollen using DNA recombination systems could be technically challenging because efficiencies of previously described DNA recombination systems tested in higher plants are not low for large-scale crop production. In the case of pollen, for example, even if excision efficiency reaches 99.99%, meaning only 0.01% of pollen grains are transgenic, there will be too many pollen grains containing transgenes under large-scale, field conditions to cause a concern. Our technology, which is the first one to use DNA recombination systems to produce transgene-free pollen and also exceptionally highly efficient, should be applicable for large-scale crop production systems. Currently the only other technology that could be used to produce transgene-free pollen is chloroplast transformation but it has been recently demonstrated that DNA can be transferred from the chloroplast and integrated into the nucleus[30]. Our system provides an excellent alternative to address the pollen-mediated transgene pollution problem of field-grown GM crops

The high efficiency of deletion of all functional transgenes from the pollen genome of host plants harboring 1-2 copies of transgenes has been confirmed with three different techniques: Southern blot hybridization, PCR amplification and histochemical assay for GUS activity. PCR amplification technique offers a highly sensitive method to detect residual transgenes while Southern blot hybridization analysis provides verification for the absence of transgenes in progenies. Histochemical assay of GUS activity, the most highly sensitive method to detect the presence of transgenes in our case, further confirms 100% deletion of transgenes from pollen grains based on large numbers of progeny seedlings resulted from the crosses of LFBF-GN lines (pollen parent) and wildtype (seed parent). Regarding the sites of excision, the DNA sequencing analysis reveals that the FLP- or Cre-recombinase-mediated deletion takes place within the two *loxP-FRT* direct repeats as expected. Because the "gene-deletor"

technology is capable of excising all functional transgenes with only a short non-native, non-protein encoding *loxP-FRT* sequence (as few as 86-bp in length), left in the pollen genome, the potential environmental and health effects of this non-expressed DNA sequence, if any, would be minimal or relatively easy to determine. On the other hand, in the near future it could also be possible to remove all foreign DNA sequences including the *loxP-FRT* recognition sequence using a recombinase that recognizes related native plant sequences or cuts outside of their recognition sequence[10, 14].

Our data suggest that the combined use of the *loxP-FRT* sequences as flanking sites might effectively improve the excision efficiencies of both Cre and FLP recombinases because excision efficiencies of the previously described Cre or FLP systems in the literature are generally low in higher plants[12, 13]. However, we realize that additional experimental data are needed to make a conclusive statement regarding the synergistic effect of the *loxP* and *FRT* sequences. In case that assumption is true, it is not clear how the combined use of the *loxP-FRT* sequences as flanking sites enhances the excision efficiencies of FLP and Cre recombinases. One possible explanation is that the hybrid *loxP* and *FRT* sequence may facilitate the formation of the hairpin structure of the *loxP-FRT* or stabilize such a structure, which is important for the recombinase-mediated excision/recombination. Another possibility is that the placement of *loxP* on one side and *FRT* on the other side as flanking sites may enhance the efficiency of the recombinase-mediated excision/recombination. Recently, Lauth et al.[24] reported that the use of *loxP* on one side and *FRT* on the other side with a Cre or *Flp* expression vector resulted in significantly higher rates of recombination.

The current version of the "gene-deletor" technology can be directly used in asexually (e.g. vegetatively) propagated plants important to the paper, forestry and ornamental horticulture industries to reduce the pollen-mediated transgene pollution problem. We have vegetatively propagated LFBF-GN lines 4 and 9 plants and observed 100% efficiency of excision of all functional transgenes in pollen of the vegetatively-propagated progenies. For sexually propagated crops, where hybrid or non-hybrid seeds are required for crop production, the "gene-deletor" technology could be applicable with a modification. In that case, an introduction of a non-phytotoxic chemical-inducible antisense FLP gene into the *loxP-FRT*-FLP system may conditionally repress the expression of the FLP gene in pollen upon the application of the inducer when seed companies are producing certified or registered seed stocks in small-scale plantation. Repression of the FLP gene expression could prevent deletion of transgenes from the pollen genome in that generation.

High efficiency of transgene deletion observed with our "gene-deletor" system provides a basis to create non-transgenic organs from transgenic organisms. One potential application is

that with a fruit-, seed- or developmental stage-specific gene promoter to control the expression of the *FLP* or *Cre* gene, we could delete all functional transgenes in seed, fruit or whole plant at a desirable time or stage of development. In the case of deleting transgenes from seed, the "gene-deletor" technology would also be useful for protection of proprietary transgenic technologies. However, the "gene-deletor" technology differs from the controversial "terminator" seed technology[25,26] that produces suicide seed and therefore prevents farmers from planting harvested seed. In contrast, farmer harvested seed from a "gene-deletor" crop should still be viable but rendered free of a patented GM trait. The "gene-deletor" technology could also be used to remove transgenes in transgenic animals from specific organs/tissues at specific developmental stages. For example, using an appropriate gene promoter to control the expression of the FLP or *Cre* gene, all foreign genes could be deleted from transgenic animals such as growth promoting or disease-resistant Salmon fish[27] several weeks before harvesting for market. In that case, functional transgenes would be removed from the host genome and their mRNA and protein products would be depleted in the animal body prior to reaching the consumer. Furthermore, the use of the combined *loxP-FRT* sequences as flanking sites for Cre or FLP recombinase might improve efficiencies of gene knockouts in animal systems.

Experimental protocol

Fusion gene construction. We synthesized a *loxP* and *FRT* recognition sequence with a spacer and a multiple cloning site (*loxP-FRT*): 5'-
gggaattc*ataacttcgtatagcatacattatacgaagttat*gaccgaagttcctatactttctagagaataggaacttcggaataggaacttcggtac
ctatgtcgacgaactcgagtagagctcaaggatccttcccggg*ataacttcgtatagcatacattatacgaagttat*gactgaagttcctatactttct
agagaataggaacttcggaataggaacttcgctagcgg-3'. The underlined italic letters represent the *loxP* site (34-bp). The underlined bold letters represent the *FRT* sequence (48-bp). The four base pairs, gacc, between the *loxP* and *FRT* sites, are a spacer sequence. The DNA sequence flanked with two *loxP-FRT* sites, ggtacctatgtcgacgaactcgagtagagctcaaggatccttcccggg, is a multiple cloning site containing *Kpn I, Sal I, Xho I, Sac I, Bam HI* and *Sma I* restriction enzyme sites. The 8-bp DNA sequence at the 5'-end contains an *EcoR I* site (gaattc) and the 8-bp DNA sequence at the 3'-end contains an *Nhe I* site (gctagc).

In order to insert the *loxP-FRT* sequence into the pBIN19 vector[16], we deleted the T-DNA sequences within the left and right borders of the vector with *EcoR I* and *Nhe I.* The synthetic *loxP-FRT* sequence digested with *EcoR I* and *NheI* was inserted into the pBIN19 vector that was also digested with *Eco RI* and *Nhe I.* The resulting pBIN19- *loxP-FRT* vector was used to host the *bgp*-FLP-*nos* gene. To amplify a 660-bp fragment of the *bgp* 5'-upsteam region[18], we used genomic DNA of *Brassica rapus* as the template, 5'-gcggtacctatcattcctttaatttcaagg-3' as the 5'-end primer, and 5'-gtctgcagttggagaggagatggggttg-3' as the 3'-end primer for a 40 cycle PCR

reaction: denaturing DNA template at 94°C for 1 min followed by 1 min for primer annealing at 58°C and then 1 min at 72°C for extension. The *Kpn I* and *Pst I*-digested 660-bp *bgp* 5'-upsteam fragment[18] was fused to the 5'-end of the FLP-*nos* sequence in a pBluescript KS II that was kindly provided by Dr. G. M. Wahl of the Salk Institute, San Diago, CA.

The *bgp*-FLP-*nos* fusion gene was excised from pBluescript KS II with *Kpn I* and *Sac I* digestion and ligated into the *Kpn I* and *Sac I* sites of the pBIN19- *loxP-FRT* vector, resulting in the pBIN19-*loxP-FRT* -*bgp*/FLP construct (pBIN19-LFBF), a gene cassette for automatic excision of LFBF from the pollen genome. We then inserted the *gusA* reporter gene and the *nptII* gene into the pBIN19-LFBF vector to produce a pBIN19-LFBF-GN construct. The 35S CaMV gene promoter-*gusA:nptII-nos* fusion gene was generated[20]. The 35S-*gusA:nptII-nos* fusion gene was excised from pBluescript KS II with *Sal I* and cloned into pBIN19-LFBF that was digested with *Sal I* to produce the pBIN19-LFBF-GN construct. The *lat52*-Cre-*nos* fusion gene was constructed using the same cloning strategy as for the *bgp*-FLP-*nos* gene. The coding sequence of the *Cre* gene was kindly provided by Dr. David Ow, USDA Plant Gene Expression Center, Albany CA[28]. The *lat52* gene promoter[23] was cloned from tomato plants using PCR-based technology with the following two specific DNA primers: 5'-ggc**ggtacc***(KpnI* site in bold) tatacccctggataag-3' and 5'-cgg**ctgcag***(Pst I* site in bold) agcacaatagcctttgcc-3'.

Plant transformation, pollination and histochemical staining. The pBIN19-LFBF-GN and pBIN19-LFLC-GN constructs were mobilized into *Agrobacterium tumefaciens* LBA 4404 and the resulting *Agrobacterium* strains were used for leaf-disk infection[29] to produce transgenic plants of *N. tabacum.* More than 35 independent transgenic lines for each construct were transferred into the greenhouse for characterization. Self-pollination and cross-pollination with wildtype and transgenic plants were preformed in the greenhouse. Flowers used for self-pollination and cross-pollination were bagged 4 days before and after pollination in order to prevent access to other pollen. Histochemical staining of GUS activity in various tissues, organs, and young seedlings was performed with the aid of vacuum infiltration of the GUS enzyme substrate[29].

Southern blot, DNA sequencing and PCR analyses of transgenic plants. Southern blot hybridization[29] was used to determine the copy number of the transgene insert in transgenic plants using 10 μg of *Eco RI, Kpn I, Hind III* and *Pst I*-digested genomic DNA of T0 transgenic and wildtype plants. The FLP or *gusA-nptII* DNA fragments were labeled with ^{32}P using the RadPrime random primer labeling kit (GibcoBRL). The hybridization signals were visualized using a Packard Cyclone Storage Phosphor System.

The presence of the transgene inserts in the genome of transgenic plants was determined by PCR. Genomic DNA from immature pollen grains (stage 7 flowers) of T0 transgenic plants was used to establish the pre-excision signal and genomic DNA from seedlings of transgenic plants (pollen parent) x wildtype (seed parent) crosses was used to establish the post-excision signal. Two oligos, 5'-gaacgtggcgagaaaggaagg-3' and 5'-actgacagaaccgcaacgttg-3', specific to the T-DNA sequences outside the LFBF-GN or LFLC-GN sequences were used as primers for PCR reactions. PCR reactions were carried out for 40 cycles at 94° C for 1 min, and 60° C for 1 min for and then 72° C for 8 minutes.

DNA fragments amplified by PCR for sequencing analysis were separated and purified on agarose gel and then eluted. DNA sequences were determined by dye terminator cycle sequencing using an Applied Biosystem 377 DNA sequencer (Perkin-Elmer Corp) at the University of Connecticut. Sequencing from both the sense and antisense orientations was performed for confirmation.

For RT-PCR reactions, 2 μg of total RNA isolated from pollen grain of stage 10 flowers were used to synthesize cDNAs with a cDNA synthesis kit (Life Technologies). One twentieth of volume of the first strand cDNA reaction was used for PCR reactions to amplify the FLP and actin cDNAs. The PCR conditions were 94° C for 1 min, 62° C for 1 min and 72° C for 1 min, 40 cycles in total. The primers, 5'-gttcgaatcggaagaagc-3' and 5-gcttgtctttgtctctgtcac-3', were used to amplify the FLP cDNA. The primers, 5'-atgccctcccacatgctatt-3' and 5'-*aaca*tggtagagccactg-3', were used for amplification of the actin cDNA.

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Figure Legends

Fig. 1 A schematic illustration of the "gene-deletor" cassette and the principle of the "gene-deletor" technology. Within the two T-DNA border sequences (LB and RB), two sites of 86-bp *loxP-FRT* recognition sequences in direct orientation are used to flank all transgenes including the FLP recombinase gene. 35S-*gusA:nptII-nos* is a fusion of the *gusA* reporter gene and the kanamycin resistance gene under the control of the 35S CaMV gene promoter. B*gp*-FLP-*nos* is a pollen specific *bgp* promoter-FLP recombinase fusion gene. Expression of the FLP gene in pollen leads to deletion of all DNA sequences within the two *loxP-FRT* sites from the pollen genome and leaves one *loxP-FRT* site behind.

Fig. 2. Southern analysis of LFBF-GN transgenic plants. Lane MM: DNA size markers. F2, F4, F6, F9 and F11: *Hind III*–digested genomic DNA isolated from T0 LFBF-GN transgenic lines 2, 4, 6, 9 and 11, respectively. Lane WT: *Hind III*-digested

genomic DNA of a wildtype plant. This analysis shows that LFBF-GN line 2 contains multiple copies of the LFBF-GN cassette, lines 4, 6 & 9 contain a single copy, and line 11 a double copy. A FLP DNA fragment was used as hybridization probe.

Fig. 3. Histochemical visualization of GUS enzyme activity (blue color) in various plant organs. (**A-D**) GUS activity was detected in leaf, stem, root and flower of a T0 LFBF-GN plant but not in mature pollen. (**E** and **F**) GUS activity was detected in immature and mature pollen grains of a 35S-*gusA* plant lacking the "gene-deletor" cassette. (**G** and **H**) GUS activity was observed in immature pollen grains but not in mature pollen grains of LFBF-GN plants. Below each flower are stained pollen grains from that flower.

Fig. 4. Molecular characterization of pollen-specific DNA excision in transgenic tobacco plants. (**A**) PCR analysis of genomic DNA prepared from transgenic plants and their progeny. MM: DNA size marker. wt-A: DNA isolated from immature pollen of wildtype flowers (stage 7 flowers). wt-B: Template DNA isolated from seedling progeny of a wildtype plant. Lanes F2-A, F4-A, F6-A, F9-A and F11-A: Template DNA isolated from immature pollen of flowers (stage 7 flowers) of LFBF-GN transgenic lines 2, 4, 6, 9 and 11, respectively. Lanes F2-B, F4-B, F6-B, F9-B and F11-B: Template DNA isolated from seedling progeny of LFBF-GN lines 2, 4, 6, 9 and 11 (pollen parent) x wildtype (seed parent) crosses, respectively. Lanes C5-A and C8-A: Template DNA isolated from immature pollen of LFLC-GN line 5 and 8 flowers (stage 7 flowers). Lanes C5-B and C8-B: Template DNA isolated from seedling progeny of LFLC-GN lines 5 and 8 x wildtype plant crosses, respectively. The 7.3-kb DNA fragment visualized contains the entire sequence of the LFBF-GN cassette and the 7.2-kb DNA fragment visualized in lanes C5-A and C8-A represents the entire sequence of the LFLC-GN cassette. The 0.2-kb DNA fragment observed in lanes B contains the 86 bp *loxP-FRT* sequence plus the T-DNA sequence left in the pollen genome after deletion. **(B):** Southern blot analysis confirms the deletion of DNA sequences flanked within the two *loxP-FRT* sites. Lanes F4 and F6: *Hind III*–digested genomic DNA isolated from T0 LFBF-GN transgenic lines 4 and 6, respectively. Lanes F4 x WT, F6 x WT, F9 x WT and F11 x WT: *Hind III*–digested genomic DNA isolated from hybrid seedlings produced from crosses of LFBF-GN transgenic lines 4, 6, 9 and 11 x wildtype plants (seed parent), respectively. Lane WT: *Hind III*-digested genomic DNA of a wildtype plant. A FLP DNA fragment was used as hybridization probe. This analysis demonstrates that the FLP gene was absent in hybrid progeny produced from crosses of LFBF-GN transgenic lines 4, 6, 9 and 11 x wildtype plants. **(C):** DNA sequence of the 0.2-kb fragment cloned from hybrid seeds produced from crosses of LFBF-GN (pollen parent) x wildtype (seed parent) plants. The DNA sequence of the 0.2-kb post-excision signal indicates that all function transgenes were deleted between the *loxP-FRT* flanking sequences in pollen and after deletion there were only one set of the *loxP* and *FRT* sequences plus some nonfunctional T-DNA sequences left in the pollen genome of the transgenic plants.

Fig. 5. RT-PCR analyses of FLP Transcript levels in pollen of LFBF-GN plants. Lane MM: A DNA size marker. Lanes F2, F4, F6, F9, F11 and WT: RT-PCT products using cDNA templates derived from pollen grains of stage 10 flowers of LFBF-GN lines 2, 4, 6, 9, 11 plants and a wildtype plant, respectively. The 0.4-kb band represents an actin cDNA fragment (an internal standard) and the 0.3-kb band represents a FLP cDNA fragment. Note the relatively weak RNA signal from the LFBF-GN line 2 (lane F2) that contained multiple copies of the LFBF-GN inserts.

Table 1. Efficiency of Transgene Deletion in Pollen with the "Gene-Deletor" Technology

Transgenic Line	Number of Seedling Progeny Examined	
	GUS(+)	GUS (-)
35S-*gusA* without the "gene-deletor" cassette:		
35S-*gusA* line 1 (pollen parent) x wildtype (seed parent)	3,054	3,301*
Single or double copies of the LFBF-GN "gene-deletor" cassette:		
LFBF-GN line 4 (pollen parent) x wildtype (seed parent)	0	25,883**
LFBF-GN line 6 (pollen parent) x wildtype (seed parent)	0	9,904**
LFBF-GN line 9 (pollen parent) x wildtype (seed parent)	0	22,057**
LFBF-GN line 11 (pollen parent) x wildtype (seed parent)	0	16,989**
Multiple copies of the LFBF-GN "gene-deletor" cassette:		
LFBF-GN line 2 (pollen parent) x wildtype (seed parent)	2,016	9 ,057***
Single copy of the LFLC-GN "gene-deletor" cassette:		
LFLC-GN line 5 (pollen parent) x wildtype (seed parent)	0	18,326**
LFLC-GN line 8 (pollen parent) x wildtype (seed parent)	0	23,305**

*GUS (-) seedlings produced from the 35S-*gusA* line without the "gene-deletor" technology are due to genetic segregation of the *gusA* gene because the transgenic plant contains a single copy of the heterozygous *gusA* gene.
** 100 % excision efficiency for deleting the *gusA* gene and other functional transgenes from the pollen genome.
*** Inefficient removal of the *gusA* gene and other functional transgenes from the pollen genome.

Fig.3

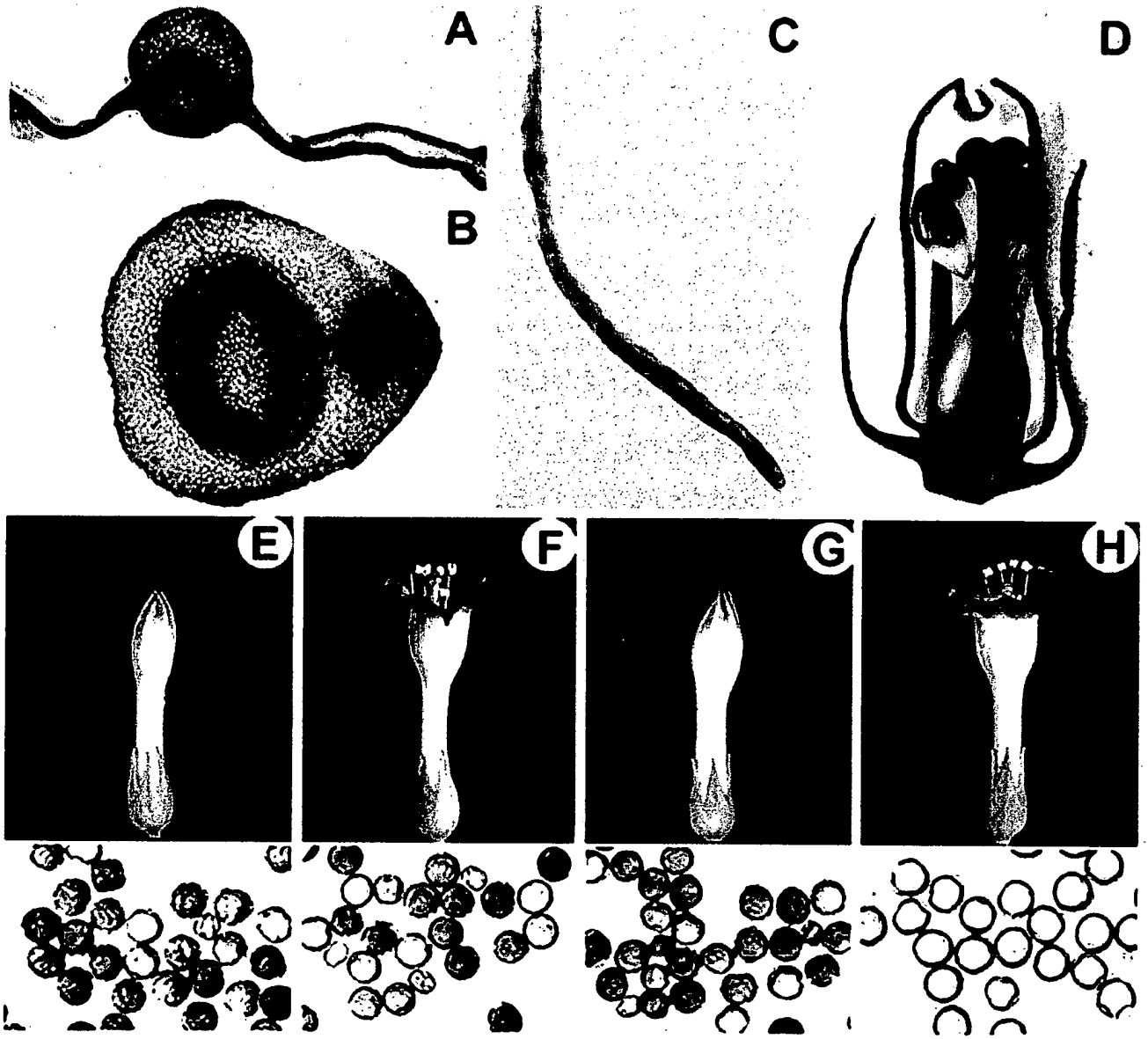


Fig. 4

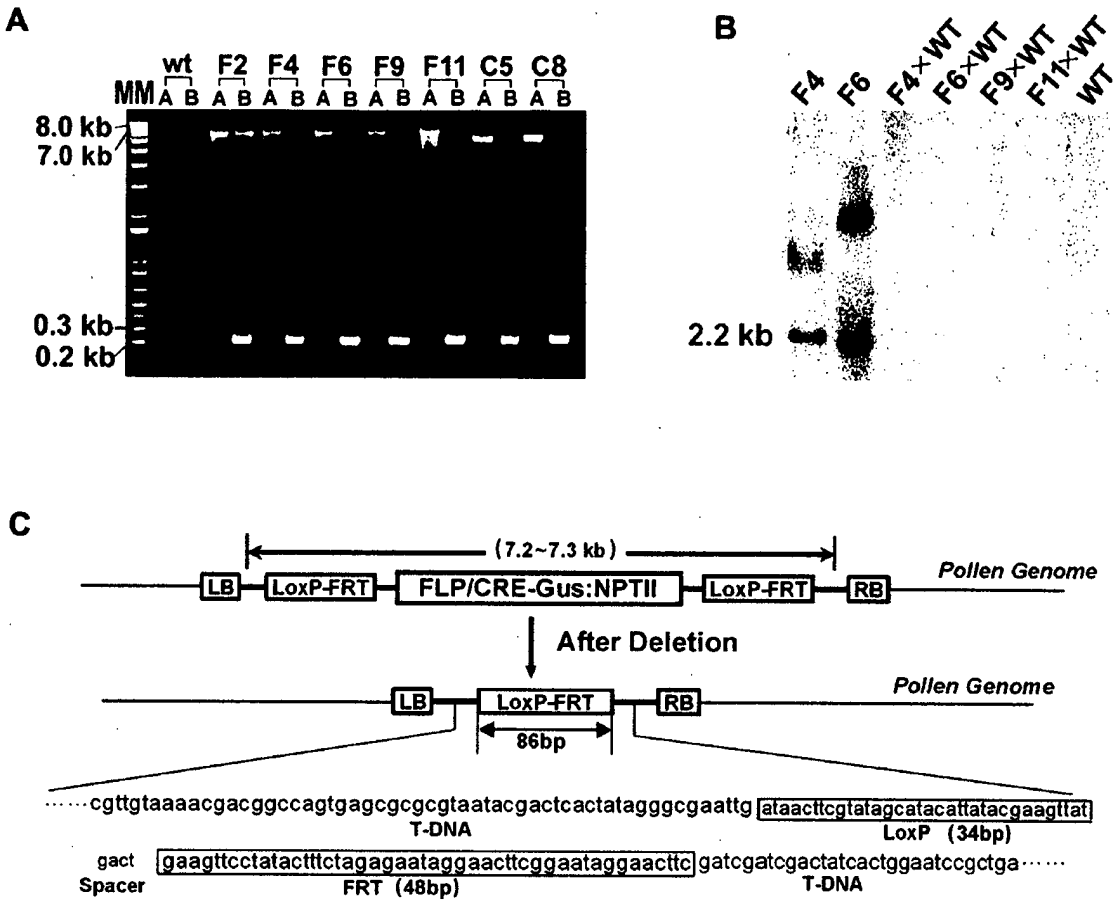
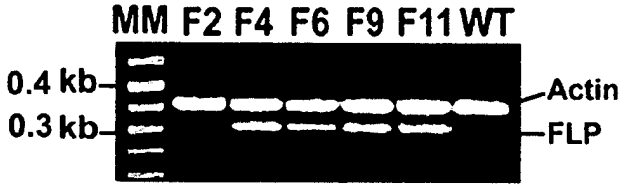

A
wt F2 F4 F6 F9 F11 C5 C8
MM A B A B A B A B A B A B A B A B
8.0 kb
7.0 kb
0.3 kb
0.2 kb
B
F4
F6
F4×WT
F6×WT
F9×WT
F11×WT
WT
2.2 kb
C
(7.2~7.3 kb)
LB
LoxP-FRT
FLP/CRE-Gus:NPTII
LoxP-FRT
RB
Pollen Genome
After Deletion
LB
LoxP-FRT
RB
Pollen Genome
86bp
·····cgttgtaaaacgacggccagtgagcgcgcgtaatacgactcactatagggcgaattg ataacttcgtatagcatacattatacgaagttat
T-DNA
LoxP (34bp)
gact gaagttcctatactttctagagaataggaacttcggaataggaacttc gatcgatcgactatcactggaatccgctga·····
Spacer
FRT (48bp)
T-DNA

Fig. 5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0.4 kb
0.3 kb
Actin
FLP